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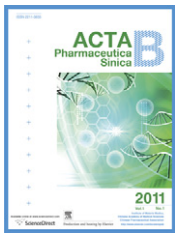

ORIGINAL ARTICLE

Catalytic cleavage activities of 10–23 DNAzyme analogs functionalized with an amino group in its catalytic core

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KEY WORDS

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8-Aza-7-deaza-2′-deoxy-adenosine;
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Chemical modification

Abstract Functionalization of the catalytic loop of 10–23 DNAzyme with an amino group was performed by incorporation of 7-(3-aminopropyl)-8-aza-7-deaza-2′-deoxyadenosine in different single positions. Among the nine modified positions in the catalytic loop, A9 is the unique position with positive contribution by such modification. These results indicated that more efficient deoxyribozymes remain to be explored by introduction of exogenous functional groups in an appropriate position in the catalytic loop of 10–23 DNAzyme, such as the combination of 7-functional group substituted 8-aza-7-deaza-2′-deoxyadenosine analogs and A9 position.

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1. Introduction

10–23 DNAzyme, an unnatural DNA enzyme obtained by *in vitro* selection from a DNA reservoir, is a sequence-specific RNA-cleaving DNA molecule[1,2]. Compared with ribozymes, its high chemical and biological stability and convenient synthesis seem to be more in favor of therapeutic and other biotechnological applications. Indeed, many efforts have been made for its applications, including the suppression of specific disease-related genes[3–16], nucleic acid structural and functional analysis, and other applications like DNA nanomachine[17–24]. Improvement of its intrinsic characteristics, such as cleavage site preferences, cleavage efficiencies, and high-level Mg^{2+}-dependence, as well as nuclease stability and efficient delivery for its optimal activities has been the focuses of many researches[25–34]. Some DNAyzmes with protein-like functional groups were selected *in vitro*, which could conduct RNA-cleavage reaction with M^{2+}-independence[29,34–37]. 10–23 DNAyzme analogs with M^{2+}-independence was obtained, but with a sacrifice of cleavage efficiency. Because an insight into the catalytic mechanism of the DNAzyme is not yet available[38,39], new random selections and chemical modifications for more efficient variants are still currently necessary[40–44]. As we have reported, screening with 7-substituted 8-aza-7-deaza-2′-deoxyadenosine derivatives in the positions of five dA residues (A5, A9, A11, A12, and A15) led to the finding that A9 is the right position for 7-substituted 8-aza-7-deaza-deoxyadenosine analogs, by which N8 atom, 7-subustituted phenethyl, 3-hydroxypropyl and 3-aminopropyl were introduced and positive effect on cleavage rate was always observed (Fig. 1)[45]. Especially, replacement of 7-(3-aminopropyl)-8-aza-7-deaza-2′-deoxyadenosine (**1**) at A9 position promised a 12-fold increase of cleavage rate. We postulated that the location of the amino group by this modification contributed to a most favorable catalytic conformation. In this study, the effect of this compound in other four non-dA positions were explored, with the aim to evaluate an appropriate position for the positive role of the extra amino group of compound **1** in the catalytic core of 10–23 DNAzyme.

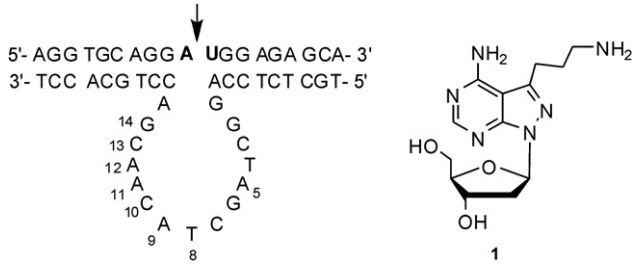

Figure 1 Left: the complex of 10–23 DNAzyme and its DNA–RNA–DNA substrate. Bold letters represent the RNA residues in the substrate. The arrow denotes the cleavage site on the substrate. Right: compound **1**.

2. Results and discussion

The oligodeoxynucleotides were synthesized with phosphoramidite chemistry. The phosphoramidite of compound **1** was synthesized according to the published procedure[45]. The oligodeoxynucleotides were purified by preparative denaturing 20% polyacrylamide gel electrophoresis (containing 7 M urea) and desalted with SEP-PAK cartridges. Characterization was performed with MALDI-TOF MS (Table 1). In the 10–23 DNAzyme analogs, compound **1** was incorporated into four positions, instead of T8, C10, C13 and G14, namely, **DZ-1-8**, **DZ-1-10**, **DZ-1-13** and **DZ-1-14** were obtained, respectively.

In order to demonstrate whether these modified DNAzymes could bind the substrate as 10–23 DNAzyme, the T_m of the complexes of these modified DNAzymes with the substrate was evaluated under the conditions for the cleavage reaction (50 mM Tris–HCl, 2 mM Mg^{2+})[45], with the chimeric DNA–RNA–DNA substrate replaced by its corresponding full-DNA substrate D19, 5′-d (AGG TGC AGG ATG GAG AGC A)-3′ to avoid any cleavage reactions conducted by DNAzyme itself and nucleases in the environment. All the complexes have a T_m of 51 °C (Table 1), very close to that of the complex of 10–23 DNAzyme and D19 (52 °C). It meant that the chemical modifications on the catalytic core could not result in changes on the thermal stability of the complexes. Furthermore, the temperature of all the cleavage reactions was set at 37 °C, much lower than the T_m of DNAyzme-substrate complexes. Therefore, it was suggested that the changes of cleavage behavior of the modified DNAzymes could be attributed to the influence of the chemical modifications on the cleavage step, instead of the thermal stability of the complex. The cleavage reaction of these DNAzymes on the ^{32}P-labled target DNA–RNA–DNA substrate was evaluated under single-turnover conditions.[45]

It has been demonstrated that compound **1** at A9 position of 10–23 DNAzyme contributed a 12-fold increase of cleavage rate, **DZ-1–9** with k_{obs} of 0.0037 min^{-1} [45]. **DZ-1–9** is the fastest DNAzyme in the screening of five dA residues with compound **1** in the catalytic core of 10–23 DNAzyme. Instead of these five dA positions, other residues in the catalytic core were substituted by compound **1** to explore the possible positive role of the extra amino group. Firstly, a position shift of compound **1** around A9 was conducted. When compound **1** in **DZ-1–9** was shifted to its 5′-residue position T8 (**DZ-1–8**), as shown in Fig. 2, **DZ-1–8** could also conduct the reaction like the parent 10–23 DNAzyme, but with a slower cleavage rate (0.0020 ± 0.0002/min) (Table 2). This replacement resulted in moderate loss of activity, compared with 10–23 DNAzyme, which was not so consistent with previous observation that T8 is the least conserved residue, any other canonical residue or even modified residue replacement results in little effect on the cleavage of 10–23 DNAzyme[40,41]. On the other hand, the replacement of compound **1** at its 3′-positioned C10 led to a further decrease of the reaction rate, as

Table 1 MALDI-TOF MS data of 10–23 DNAzyme analogs.

DNAzyme	Sequences (5′–3′)	MW (calcd.)	MW (found)
DZ-1-8	d(tgc tct cca GGC TAG C**1**A CAA CGA cct gca cct)	10,060.5	10,062.5
DZ-1-10	d(tgc tct cca GGC TAG CTA **1**AA CGA cct gca cct)	10,075.6	10,077.4
DZ-1-13	d(tgc tct cca GGC TAG CTA CAA **1**GA cct gca cct)	10,075.6	10,077.0
DZ-1-14	d(tgc tct cca GGC TAG CTA CAA C**1**A cct gca cct)	10,035.6	10,036.6

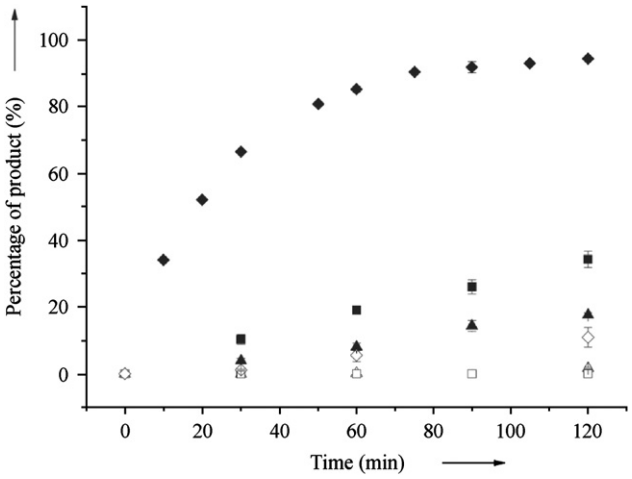

Figure 2 The position-dependent effect of compound **1** in the catalytic loop of 10–23 DNAzyme: at A9 (**DZ-1-9**, solid diamond)[45], at T8 (**DZ-1-8**, solid triangle), at C10 (**DZ-1-10**, open diamond), at C13 (**DZ-1-13**, open square), at G14 (**DZ-1-14**, open triangle), and 10–23 DNAzyme (solid square)[45].

Table 2 Observed rate constants of 10–23 DNAzyme and its analogs measured under single-turnover conditions.

DNAzyme	T_m (°C)	k_{obs} /min
10–23 DNAzyme	52	0.0037 ± 0.00007[45]
DZ-1-9	51	0.045 ± 0.004[45]
DZ-1-8	51	0.0020 ± 0.0002
DZ-1-10	51	0.0012 ± 0.0001
DZ-1-13	51	ND
DZ-1-14	51	ND

ND: not detected under present conditions.

observed with **DZ-1–10** (0.0012 ± 0.0001 min^{-1}). It meant that the occupation of cytosine at this position was necessary[38]. Up to now, deletion of C10 or its replacement with other natural or modified residues always resulted in moderate to significant loss of cleavage activity.

The amino groups of C13 and G14 have been demonstrated to be very important for the function of 10–23 DNAzyme[38], their amino groups are supposed to be involved in the reaction in some way. When an extra amino group was introduced to these two positions by compound **1**; however, such changes led to a significant loss of the catalytic function, as shown by **DZ-1–13** and **DZ-1–14** (Fig. 2). It seems that the new interactions related to compound **1** at these two positions was detrimental to the reaction, other functional groups in C13 and G14 as well as their spacial occupation might be important for their roles in the context of the catalytic loop.

As demonstrated in our work, in the total nine modified positions (A5, T8, A9, C10, A11, A12, C13, G14 and A15) in the catalytic core of 10–23 DNAzyme[45], the effect of compound **1** was position-dependent. These observations emphasized the importance of the location for the extra amino group from compound **1** and the conservation of the residues defined by *in vitro* selection. In other words, the catalytic core of 10–23 DNAyzme is very sensitive to chemical modifications, no matter more or less catalytic power of the new DNAzymes. More importantly, we learnt from these results that A9 is the most appropriate position for the introduction of 7-substituted 8-aza-7-deaza-2′-deoxyadenosine analogs, up to now. This screening research promised more efficient deoxyribozymes for practical applications by chemical functionalization at A9 in the catalytic core of 10–23 DNAzyme.

3. Experimental

3.1. Oligodeoxynucleotides

Oligodeoxynucleotides were synthesized on an ABI 392 DNA/RNA synthesizer (Applied Biosystems, USA) with the DMT-off mode, on a 1 μmol scale according to the User Protocol. The phosphoramidites of canonical residues were purchased from Proligo (Sigma-Aldrich). The oligodeoxynucleotides were deprotected in conc. aq. ammonia for 18 h at 60 °C and purified by a 20% polyacrylamide/7 M urea denaturing gel. After desalting with SEP-PAK cartridges (Oasis MAX, C18, Waters, USA), the product was characterized by MALDI-TOF performed on an autoflex TOF/TOF (BRUKER, Daltonics) with HPA as the matrix. The chimeric substrate was purchased from Takara (Dalin, China). T_m measurement was conducted on a Cary-100 Bio UV–Visible spectrophotometer equipped with a Cary temperature controller (Varian, USA)[45].

3.2. Cleavage reaction under single-turnover conditions[45]

Prior to the cleavage reaction, the solution of deoxyribozyme (2 μM) and the ^{32}P-labeled substrate[45] (20 nM) in 50 mM Tris–HCl (pH 7.4) was denatured at 90 °C for 3 min and cooled to 37 °C. Mg^{2+} was added to a final concnentration of 2 mM, and the cleavage reaction was initiated. At different time points, aliquots were taken from the reaction mixture and quenched by an equal volume of stopping solution (100 mM EDTA, 8 M Urea). The substrate and the cleavage product in the samples were separated by electrophoresis on a 20% polyacrylamide gel containing 7 M urea, and quantified by the densitometry of the gel images with a Molecular Dynamics Storm 840 Phosphoimager. The observed rate constant k were calculated according to the equation $P\% = P_\infty\% - C \cdot \exp[-k_{obs}t]$, where $P\%$ is the cleavage percentage of product at time t, $P_\infty\%$ is the final percentage of the product at $t = \infty$, C is the difference in $P\%$ between $t = \infty$ and $t = 0$. The data was given as the averaged results of three independent experiments. Less than 20% variation was observed for identical experiments performed on different days.

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